



Research Paper

Vinyl chloride-induced interaction of nonalcoholic and toxicant-associated steatohepatitis: Protection by the ALDH2 activator Alda-1

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ARTICLE INFO

Keywords:

Vinyl chloride

PVC

Angiosarcoma

Nonalcoholic fatty liver disease

Toxicant-associated steatohepatitis

Aldehyde dehydrogenase

ABSTRACT

Vinyl chloride (VC), an abundant environmental contaminant causes steatohepatitis at high levels, but is considered safe at lower (i.e., sub-OSHA) levels. However, we have previously shown that even lower VC levels exacerbate experimental nonalcoholic fatty liver disease (NAFLD) caused by high-fat diet (HFD). Mitochondrial oxidative injury and subsequent metabolic dysfunction appeared to play key roles in mediating this interaction. Mitochondrial aldehyde dehydrogenase 2 (ALDH2) serves as a key line of defense against endogenous and exogenous reactive aldehydes. The current study therefore tests the hypothesis that allosteric activation of ALDH2 with Alda-1 will protect against VC-enhanced NAFLD. Mice were exposed to low VC concentrations (< 1 ppm), or room air for 6 h/day, 5 days/week for 12 weeks, while on HFD or low-fat control diet (LFD). Some mice received Alda-1 (20 mg/kg i.p., 3 × /week) for the last 3 weeks of diet/VC exposure. Indices of liver injury, oxidative stress, metabolic and mitochondrial (dys)function were measured. As observed previously, low-dose VC did not cause liver injury in control mice; while liver injury caused by HFD was enhanced by VC. VC decreased hepatic ALDH2 activity of mice fed HFD. Alda-1 attenuated oxidative stress, liver injury, and dys-metabolism in mice exposed to HFD + VC under these conditions. Importantly, alterations in mitochondrial function caused by VC and HFD were diminished by Alda-1. Previous studies have indicated that liver injury caused by HFD is mediated, at least in part, by enhanced mitochondrial autophagy (mitophagy). Here, Alda-1 suppressed PINK1/PARKIN-mediated mitophagy. Taken together, these results support the hypothesis that ALDH2 is a critical defense against mitochondrial injury caused by VC in experimental NAFLD. The ALDH2 activator Alda-1 conferred protection against liver damage under these conditions, most likely via increasing clearance of aldehydes and preserving mitochondrial respiratory function.

1. Introduction

Vinyl chloride (VC) gas is a volatile organic compound that is used in industry to create the polymer, polyvinyl chloride (PVC), and its global production was recently estimated at 27 million metric tons annually [1]. In addition to occupational exposure, environmental exposure to VC is common. Indeed, a recent study indicated that neonates have already adult exposure levels to VC and other VOCs [2]. VC is a common contaminant in groundwater surrounding industrial sites and Superfund sites. VC readily volatilizes from water sources and thereby can suffuse into homes [3–5]. Environmental exposure to VC may

actually increase in the near future, as VC is a common solvent found in natural gas fracking fluids [6,7]. Owing to its widespread environmental presence and its known potential human risk, VC is ranked #4 on the ATSDR Hazardous Substance Priority List [8]. The risks of VC exposure to human health are incompletely understood.

High occupational exposure levels of VC directly causes toxicant-associated steatohepatitis (TASH) with necrosis, fibrosis, and cirrhosis [9,10], as well as hepatocellular carcinoma (HCC) and the otherwise extremely rare hepatic hemangiosarcoma [11]. Due to these direct toxicity concerns, the Occupational Safety and Health Administration (OSHA) has decreased the acceptable level of VC exposure to < 1 ppm

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<https://doi.org/10.1016/j.redox.2019.101205>

Received 20 March 2019; Received in revised form 10 April 2019; Accepted 14 April 2019

Available online 19 April 2019

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[4]. However, the toxicity of environmental exposure below this OSHA limit, but higher than the EPA reference concentration (RfC) [4] is unclear. Moreover, the integrated risk of VC exposure with other factors and/or underlying liver diseases is not known [12]. This lack of understanding is especially important, considering the rapidly growing global burden of nonalcoholic fatty liver disease (NAFLD) [13–15]. Recent studies by our group have shown that VC exposure levels that are not directly hepatotoxic (< 1 ppm), enhanced liver damage caused by experimental NAFLD in mice [16]. This interaction was characterized by altered metabolism, inflammation and oxidative stress [16]. VC exposure also enhanced mitochondrial dysfunction caused by experimental NAFLD [16], which is thought to actually drive the other effects observed under these conditions [17,18].

Given that mitochondria have a high propensity to generate oxidative stress [19], it is surprising how relatively sensitive this organelle is to reactive oxygen species damage. ALDH2, although usually associated with acetaldehyde metabolism, is a key mitochondrial enzyme responsible for most other aldehydes, including lipid aldehydes (e.g., 4-HNE) and the VC metabolite, chloroacetaldehyde [20–22]. Indeed, in a compartment that is so exquisitely sensitive to damage, ALDH2 serves as a key line of defense against reactive aldehydes. Activation of ALDH2 has been shown to be protective in several models of oxidative stress-induced organ damage, including cardiac ischemia/reperfusion [23], pulmonary artery hypertension [24], and hepatic regeneration [25]. Importantly, it has also been demonstrated previously that ALDH activity was decreased in human NASH [26]. The purpose of the current study was to test the hypothesis that ALDH2 activation via the allosteric activator, Alda-1, will protect against the interaction between VC exposure and experimental NAFLD in mice.

2. Materials and Methods

2.1. Animals and treatment

Six weeks old male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed and exposed at 25 °C in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and procedures were approved by the local Institutional Animal Care and Use Committee. Food and tap water were allowed ad libitum. Mice were fed low-fat control (LFD, 13% fat) or high-fat diet (HFD, 42% fat) for 12 weeks. Mice were exposed to VC (Kin-tek, La Marque, TX), or room air at concentrations below the current OSHA limit ($\sim 0.85 \pm 0.1$ ppm) for up to 12 weeks (6 h per day, 5 days per week in state-of-the-art inhalation chambers) [16]. At the end of 9th week (see timeline Fig. 2A), some mice received a specific agonist of ALDH2, Alda-1 (20 mg/kg i.p.; EMD Chemicals, Gibbstown, NJ; chemical structure Fig. 2B) [25], or vehicle (50% dimethylsulfoxide and 50% polyethylene glycol). This dose of alda-1 was based on pilot studies and previously published work [25]. Body weight and food consumption were monitored weekly. At sacrifice, mice were fasted for 4 h and anesthetized with ketamine/xylazine (100/15 mg/kg, i.p.). Blood was collected from the vena cava prior to exsanguination, and citrated plasma was stored at -80 °C for further analysis. Portions of liver tissue were snap-frozen in liquid nitrogen, embedded in frozen specimen medium (Sakura Finetek, Torrance, CA), or were fixed in 10% neutral buffered formalin.

2.2. Biochemical analyses, histology, immunohistochemistry and electron microscopy (EM)

Oral glucose tolerance tests (OGTT) were performed during the 12th week of exposure. Mice were fasted for 6 h prior to OGTT. Glucose (2 mg/kg in 4 ml/kg of sterile saline solution, Sigma, St Louis, MO) was administered orally. Blood was collected from the tail vein immediately prior and at 15, 30, 60, 90 and 120 min after glucose administration. Glucose concentrations were measured using an Accu-Chek Aviva Plus

glucometer and test strips (Roche Diagnosis Corp., Indianapolis, IN) [27,28]. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by using standard kits (Thermo Fisher Scientific, Middletown, VA). Paraffin embedded liver sections were stained with hematoxylin & eosin (H&E) for hepatic morphology and neutrophil accumulation was assessed by chloroacetate esterase (CAE; Sigma, St. Louis, MO). Pathology was scored (inflammation and necrosis) in a blinded manner as described previously [29,30], the number of inflammatory and/or necrotic foci was determined in 10 400x fields. CAE positive cells were counted by Metamorph Image Analysis Software (Molecular Devices, Sunnyvale, CA) and expressed as the ratio of positive cells per 1000 hepatocytes. Adducts of malondialdehyde (MDA) in liver sections were detected by immunohistochemistry using a rabbit antibody for MDA (Alpha Diagnostic, San Antonio, TX). Hepatic MDA levels were quantified by determination of thiobarbituric acid reactive substances (TBARS) concentration using a commercially available kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Liver lipids were extracted as previously described [31]. Total triglyceride and NEFA levels were determined using standard clinical chemistry reagents (Infinity, Thermo Fisher Scientific, Middletown, VA). Liver sections were stained with Oil-Red O (ORO) for visualization of neutral lipids and Periodic Acid-Schiff (PAS) for glycogen deposition. The EM analysis was performed as described previously [32]. ALDH2 enzymatic activity was detected with a fluorometric developer using a commercially available kit (Cayman Chemical, MI). For that, hepatic mitochondria were isolated as previously described [33]. As it is difficult to measure changes in ALDH2 activity using Alda-1 in vivo, these experiments were carried out in samples from mice exposed to \pm HFD \pm VC, only.

2.3. Immunoblots

Liver samples were homogenized in RIPA buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA], containing protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Whole liver lysates were loaded onto SDS-polyacrylamide gels (Invitrogen, Thermo Fisher Scientific, Grand Island, NY), followed by electrophoresis and Western blotting onto PVDF membranes (Hybond P, GE Healthcare Bio-Sciences, Pittsburgh, PA). The membranes were washed in TBST buffer and blocked with TBST containing 5% milk. Primary antibodies against Atg7, p62, LC3II, GAPDH (Cell Signaling Technology; Beverly, MA), PINK1 and PARKIN (Santa Cruz Biotechnology, Dallas, TX) were used. Densitometric analysis was performed using UN-SAN-IT Gel (Silk Scientific, Orem, UT) software.

2.4. Isolation of primary hepatocytes and measurement of oxygen consumption rates

Primary hepatocytes, isolated from C57BL/6J mice, were plated at 10,000 cells per well on collagen-coated XF96 culture microplates (Seahorse Biosciences, Billerica, Massachusetts) and incubated at 37 °C to allow cell attachment. Cells were pre-exposed to Alda-1 at 20 μ M for 30min. For modulating mitochondrial respiration, hepatocytes were exposed to CAA (40 μ M), oligomycin (1 μ g/ml), FCCP (4 μ M) and antimycin A (10 μ M). Oxygen consumption rates (OCRs) were measured using an XF96 Extracellular Flux Analyzer (Seahorse Biosciences, Billerica, Massachusetts) [16,30]. Delta OCR was calculated by subtracting the OCR at the first measured time point after CAA addition from the OCR baseline.

2.5. ALDH2 enzymatic assay

Purified ALDH2 protein (10 μ g, Sigma, St Louis, MO) was incubated in 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT and 1 mM NAD⁺. The reaction was initiated by the addition

of CAA (40 μ M). ALDH2 activity was measured spectrophotometrically at 340 nm via the formation of NADH. For the CAA group, enzyme was exposed to CAA for 5 min, and activity was measured immediately after NAD⁺ addition. For the Alda-1 group, ALDH2 was incubated with Alda-1 (20 μ M) for 2 min and activity was measured immediately after CAA and NAD⁺ addition. Additionally, ALDH2 enzyme was pre-incubated with Alda-1 for 2 min, then exposed it to CAA for 5 min and activity was measured immediately after NAD⁺ addition. Due to an extremely fast reaction time, delta OCR was calculated by the subtracting the OCR at the first measured time point after CAA addition from basal OCR value.

2.6. Statistic analyses

Results were reported as means \pm SEM (N = 4–7) and analyzed using Sigma Plot 11.0 (Systat Software, Inc; San Joes, CA). ANOVA with Bonferroni's post host test (for parametric data) or Mann-Whitney Rank sum test were used for determination of statistical significance among treatment groups, as appropriate. A p value < 0.05 was selected before the study as the level of significance.

3. Results

3.1. VC enhances ALDH2 dysfunction caused by HFD in liver

Previous data from this group demonstrated that VC impairs mitochondrial function, likely due to an accumulation of endogenous aldehydes and/or VC-derived chloroacetaldehyde [16]. Aldehydes increase mitochondrial-derived reactive oxygen species (ROS) and decrease mitochondrial membrane potential [25]. Mitochondrial dysfunction, such as loss of mitochondrial membrane potential can trigger drastic structural changes in mitochondria from a tubular to globular shape [34]. Indeed, here VC caused dramatic changes to mitochondrial structures in mice that were concomitantly fed HFD as depicted in representative EM photomicrographs (Fig. 1A), supporting the hypothesis for a potential role of aldehydes in VC-induced liver injury. Due to its critical function in aldehyde metabolism/clearance, ALDH2 may protect the mitochondria against oxidative damage caused by aldehydes during liver injury. We therefore hypothesized that ALDH2 may also be critical during VC exposure. Hepatic mitochondria were isolated from mice exposed to VC and HFD for 12 weeks and enzymatic ALDH2 activity assays were performed for determination of aldehyde clearance rate. While VC had no effect on ALDH2 enzyme activity in the absence

of HFD, it significantly enhanced the decrease in ALDH2 enzymatic activity by HFD (Fig. 1B). These data support the hypothesis that VC may enhance HFD-induced liver injury, at least in part, via impairing ALDH2 function.

3.2. ALDH2 affects the metabolic phenotype of mice by the interaction of VC and HFD

During the course of the study (see timeline, Fig. 2A), the metabolic phenotype of the mice was monitored by determining body weights, food consumptions and oral glucose tolerance (Fig. 2). VC had no effect on the change in body weights in the LFD or the HFD groups (Fig. 2C). Body weights of mice receiving Alda-1 were significantly lighter compared to those of non-Alda-1 treated mice. Importantly, all groups consumed similar amounts of food and no significant differences were observed (Fig. 2D). Blood glucose levels of mice fed HFD were significantly higher than that of LFD control, while VC did not alter these glucose levels (Fig. 2E). However, Alda-1 significantly decreased blood glucose levels in all groups, suggesting an increase in glucose tolerance. This was also reflected in the area under the curve (AUC) with Alda-1 causing a significant decrease in OGTT in both the HFD and the HFD + VC group.

3.3. Activation of ALDH2 attenuated liver injury caused by HFD and VC

Fig. 3A shows representative photomicrographs depicting general hepatic morphology (H&E). As previously shown by this group, no overt pathological changes were observed in liver tissue by VC in the absence of HFD (Fig. 3) [16]. HFD significantly increased liver injury, as reflected in histology (Fig. 3A), elevated transaminases (Fig. 3B) and pathology scores (Fig. 3C). As previously shown, VC enhanced these effects. Importantly, Alda-1 significantly decreased liver damage, plasma transaminase activity and pathology scores (Fig. 3B and C).

3.4. ALDH2 decreased neutrophil infiltration and oxidative stress in livers of VC and HFD

Inflammation and oxidative stress are important markers of liver injury caused by the interaction of HFD and VC [16]. VC enhanced recruitment of neutrophils (CAE) caused by HFD. Alda-1 significantly reduced these markers (Fig. 4A and B). Malondialdehyde (MDA), the main byproduct of lipid peroxidation and marker of cellular oxidative

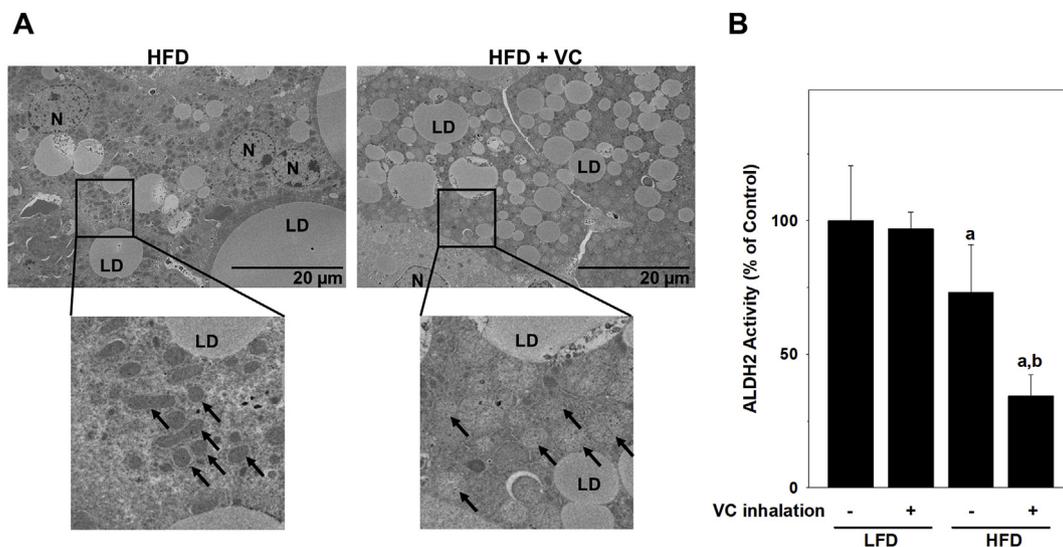


Fig. 1. ALDH2 expression and enzymatic activity. A: Representative EM photomicrographs are shown. Arrows denote for mitochondria, LD = lipid droplet, N = nucleus. B: ALDH2 activity was determined as described in Materials and Methods. ^a, p < 0.05 compared to LFD control, ^b, p < 0.05 compared to absence of VC. Samples size per group n = 8–10.

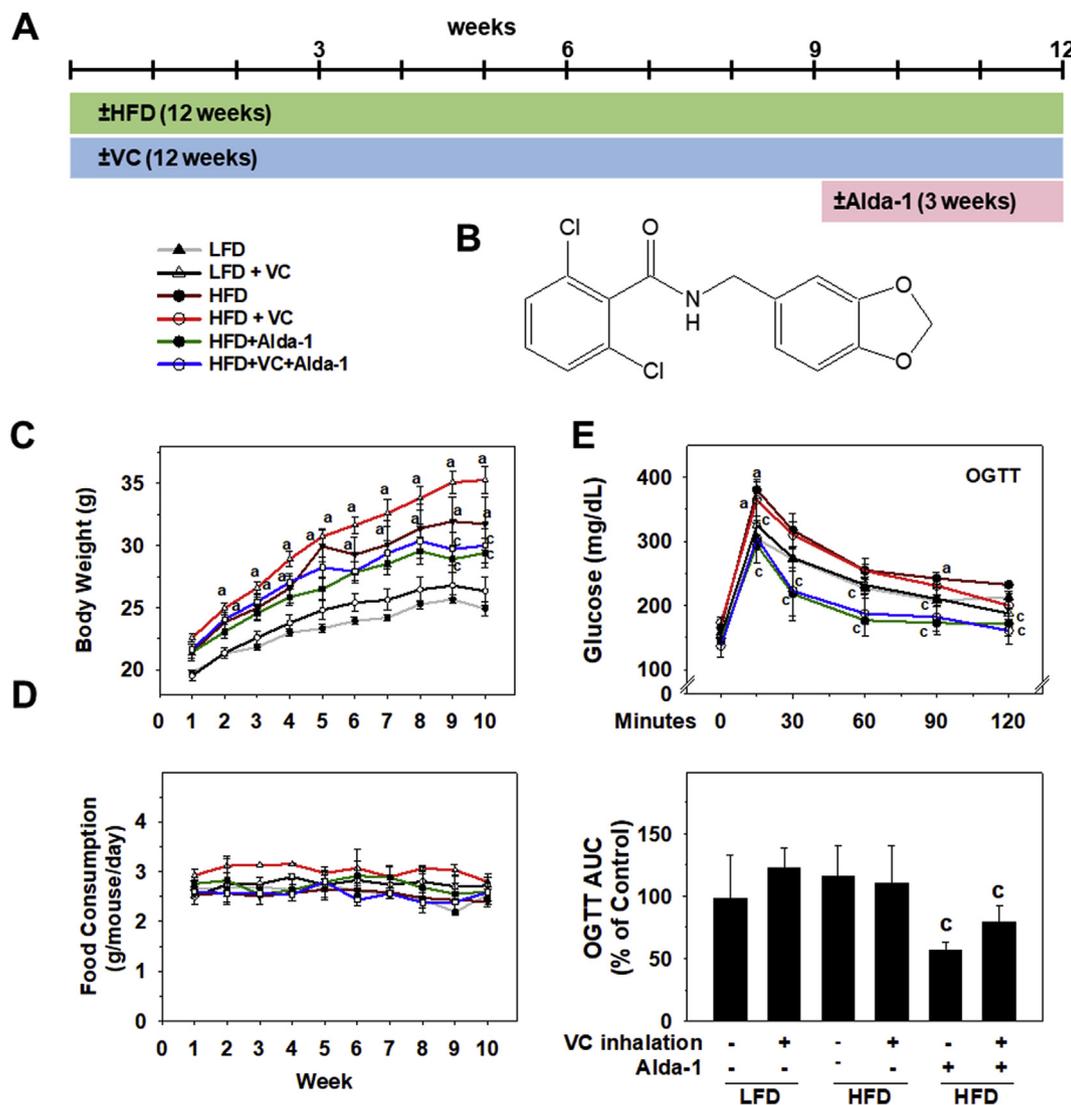


Fig. 2. Metabolic phenotype. A: Timeline of the experimental design. B: chemical structure of Alda-1. C: Body weight of mice for all groups was monitored once per week for 12 weeks. D: Food consumption was measured twice per week during the 12-week exposure period. E: Oral glucose tolerance tests (OGTT) were performed and the area under the curve (AUC) was calculated. ^a, $p < 0.05$ compared to LFD control, ^c, $p < 0.05$ compared to absence of Alda-1. Samples size per group $n = 8-10$.

stress, was increased by HFD + VC (Fig. 4A). However, Alda-1 suppressed lipid peroxidation in mice exposed to HFD + VC. These data were also reflected in a quantitative TBARS assay (Fig. 4B).

3.5. ALDH2 reversed metabolic disorders in the interaction of HFD and VC

Previously published data from this lab have shown that energy metabolism is dysregulated by the interaction of VC and HFD [16]. Lipid accumulation and glycogen storage are associated with energy metabolism in liver, disruption of carbohydrate/lipid homeostasis can potentially impact hepatic regeneration during injury and induce hepatotoxicity [35]. Liver sections were stained for ORO and PAS to evaluate hepatic metabolism (Fig. 5A). As previously observed by this group, HFD caused steatosis, and this was enhanced by VC on the level of TG accumulation (Fig. 5B). Alda-1 intervention decreased both hepatic TG and NEFA levels significantly. However, cholesterol levels were further increased by ALDH2 activation. Hepatic glycogen content as visualized by PAS staining, was normal in the LFD and the LFD + VC group. HFD and HFD + VC decreased hepatic glycogen stores, which was restored by Alda-1 administration (Fig. 5A and B).

3.6. ALDH2 decreased liver damage via participating in autophagy regulation

Autophagy is considered an adaptive process that degrades unwanted, excess and/or damaged cytosolic components in response to multiple cellular stressors including oxidative stress and pharmacological insults. Moreover, damaged mitochondria may also be subjected to a subset of autophagy, mitophagy, as a way for the cell to control mitochondrial quality [36]. Importantly, mitochondrial oxidative injury plays a key role in mediating damage caused by the interaction between VC and NAFLD [16]. Therefore, markers of autophagy (Fig. 6A and B) and mitophagy (Fig. 6A and C) were examined via Western blot analysis in this model. While HFD increased hepatic Atg7 and PARKIN protein expression, VC did not change this effect. VC increased the ratio of LC3II to LC3I in LFD and HFD mice. Alda-1 did not affect HFD + VC-induced changes in Atg7 and LC3 protein expression, but it significantly decreased p62, PINK and PARKIN protein expression.

3.7. ALDH2 protected mitochondrial function from VC metabolites

Previous work by our lab demonstrated that VC metabolite

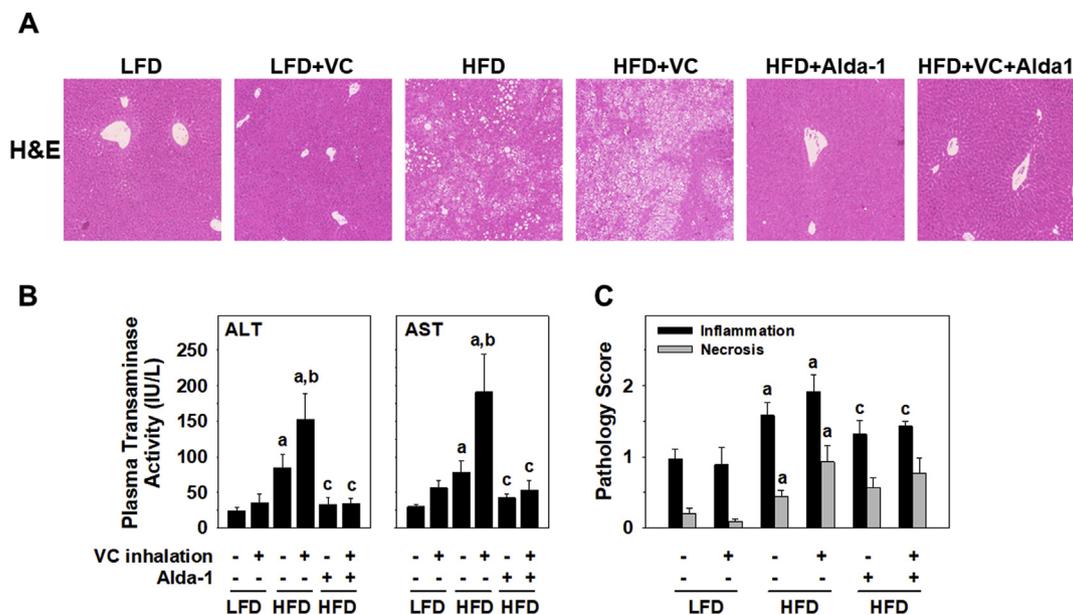


Fig. 3. Alda-1 decreased liver injury caused by HFD and VC. A: Representative photomicrographs of H&E (general morphology, 200x) are shown of mice sacrificed at 12 weeks. B: Plasma transaminase (ALT/AST) levels were determined for all the experimental groups at the 12-week time point. C: Pathology scores of all groups were evaluated as described in Material and Methods. ^a, $p < 0.05$ compared to LFD control, ^b, $p < 0.05$ compared to absence of VC, ^c, $p < 0.05$ compared to absence of Alda-1. Samples size per group $n = 8-10$.

chloroacetaldehyde (CAA) directly impaired mitochondrial membrane potential, decreased oxygen consumption rate (OCR) and mitochondrial bioenergetic reserve capacity [30]. Moreover, ALDH2 activation has been demonstrated to reverse a decrease in mitochondrial membrane potential caused by 4-HNE exposure [25]. Here, we therefore hypothesize that mitochondrial dysfunction caused by CAA that may be rescued by activation of ALDH2 with Alda-1. Seahorse bioenergetic analysis was therefore performed to assess mitochondrial function in primary murine hepatocytes (Fig. 7A and B). CAA rapidly inhibits mitochondrial function resulting in significantly increased delta OCR, demonstrating OCR inhibition immediately after CAA addition (Fig. 7A

and B). Importantly, pre-exposure to Alda-1 significantly attenuated this effect. ALDH2 plays a critical role in aldehyde clearance and thereby cellular protection [37]. Here, activity of purified ALDH2 enzyme (\pm CAA, \pm Alda-1) was measured to demonstrate the effect of VC metabolites on ALDH2 directly (Fig. 7C). CAA not only acted as substrate for ALDH2, but also blocked the conversion of NAD^+ to $NADH$, demonstrating a significant inhibition ALDH2 activity. Alda-1 pre-treatment increased enzymatic activity, both in the control and the CAA group (Fig. 7C).

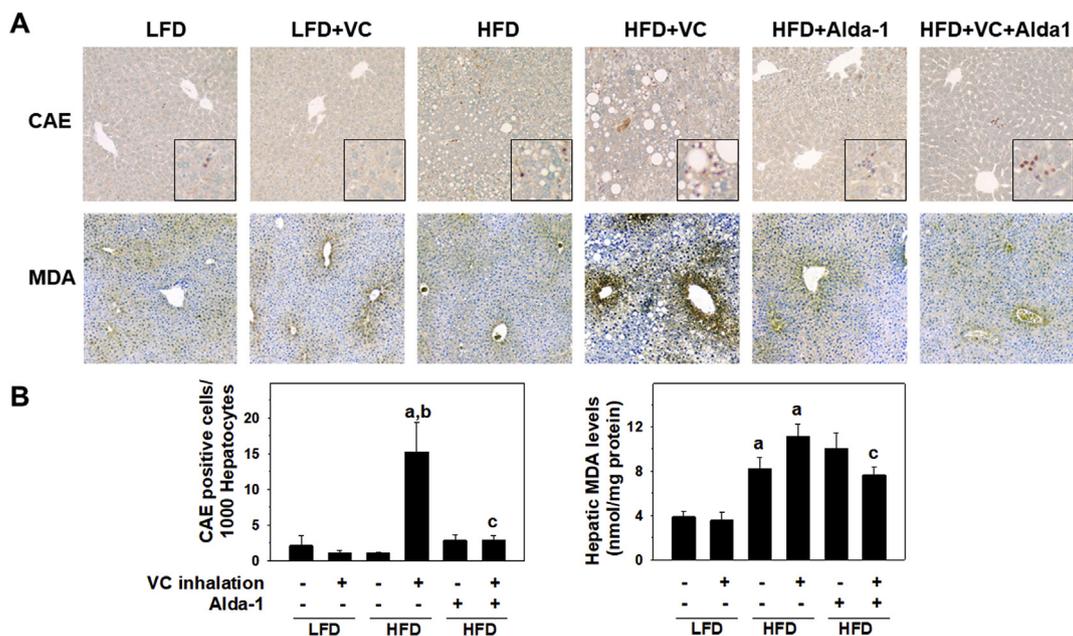


Fig. 4. Alda-1 attenuated hepatic inflammation and oxidative stress. A: Representative photomicrographs of CAE (neutrophils, purple) and MDA (index of oxidative stress, brown) are shown at 200x magnification. B: CAE-positive cells were counted and graphed as positive cells per 100 hepatocytes. Hepatic MDA levels (nmol/mg protein) were measured using a thiobarbituric acid reactive substances (TBARS) assay, as described in Material and Methods. ^a, $p < 0.05$ compared to LFD control, ^b, $p < 0.05$ compared to absence of VC, ^c, $p < 0.05$ compared to absence of Alda-1. Samples size per group $n = 8-10$.

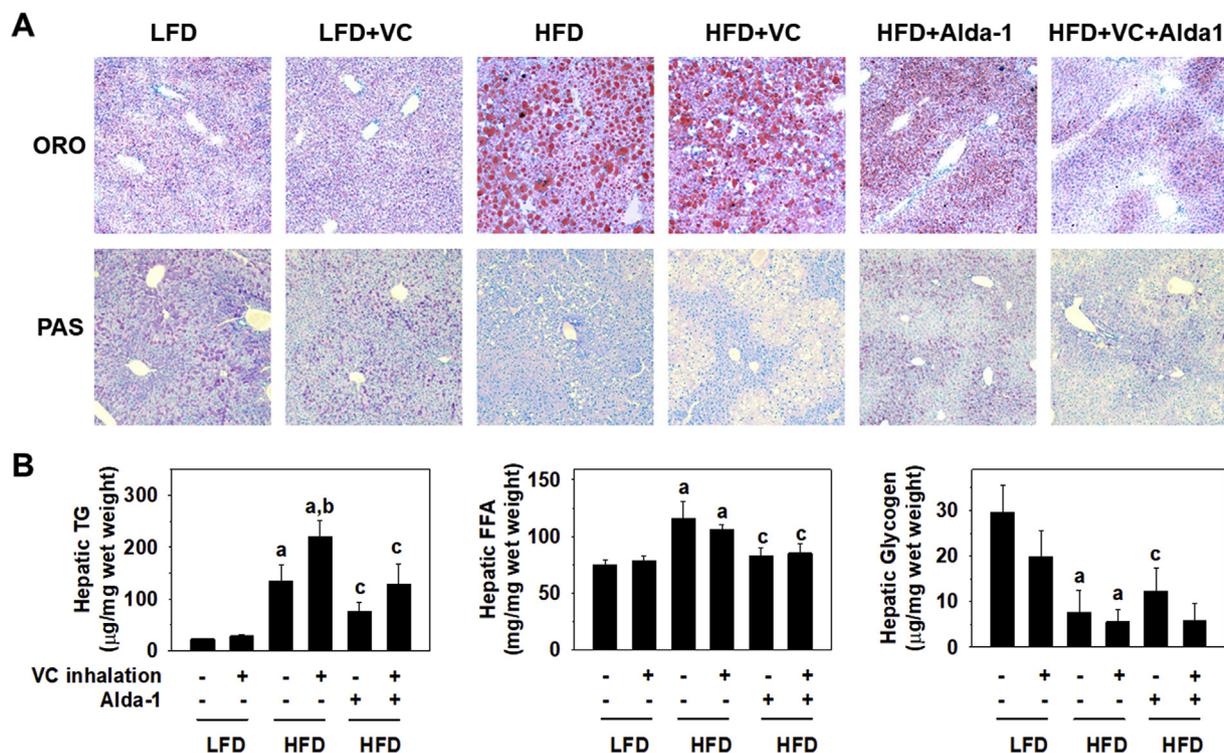


Fig. 5. Aldehyde-1 reversed metabolic dysregulation caused by HFD and VC. **A:** Representative photomicrographs of ORO (neutral lipids, red) and PAS (glycogen, dark purple) staining are shown at 200x magnification. **B:** Hepatic glycogen, TG and FFA content was measured as described in Material and Methods. ^a, $p < 0.05$ compared to LFD control, ^b, $p < 0.05$ compared to absence of VC, ^c, $p < 0.05$ compared to absence of Aldehyde-1. Samples size per group $n = 8-10$.

4. Discussion

We have demonstrated here that low concentrations of VC, in combination with HFD, impairs ALDH2 function, resulting in an accumulation of endogenous aldehydes and oxidative stress in vivo. Moreover, VC metabolite chloroacetaldehyde directly blocked ALDH2 activity in vitro. Treatment with ALDH2 activator Aldehyde-1 reversed pre-established liver injury oxidative stress and metabolic dysregulation caused by chronic (low level) VC and HFD.

OSHA has regulated occupational exposure levels to VC since the 1970's; these regulations lessen concerns about direct toxicity caused by occupational exposure to VC. However, we demonstrated that lower exposure levels of VC and metabolites that are not overtly toxic, per se, can augment liver toxicity caused by another insult [16,27,30]. Such an interaction is supported by literature precedence. Specifically, several studies have established that physiological and/or biochemical changes to the liver that are pathologically insignificant can enhance injury in response to another agent (i.e. the "2-hit hypothesis") [38,39]. Indeed, this "2-hit hypothesis" is viewed as a consistent mechanism by which liver diseases initiate and progress to more severe states and that primary risk (e.g. HFD, metabolic syndrome, etc.) is modified by additional genetic and/or environmental factors; VC may be such an environmental factor.

Our previous work suggested that mitochondria are selectively targeted by VC exposure [16,30]. Specifically, we showed VC directly damages the hepatic mitochondrial electron transport chain, even at exposure levels that cause no overt pathology [16,30]. Here, although VC exposure alone decreased oxygen consumption rates (OCR) in hepatocytes, this apparent mitochondrial injury did not translate to overt hepatic pathology (Fig. 3). Mitochondria have been identified to play key roles in hepatic (mal)adaptation to NAFLD. Although the underlying mechanisms are incompletely understood, morphological changes of the mitochondria [40,41] and mitochondrial-driven alterations in substrate supply, metabolism, and cell death have been consistently

identified as likely players. We therefore hypothesized that VC-induced mitochondrial damage may impede the liver to appropriately adapt to the biochemical stresses caused by experimental NAFLD and thereby exacerbate damage. This mitochondrial injury also correlated with an overall decrease in respiratory capacity in the mice [16,30]. There appears to be a feed-forward loop between weight gain and altered energy expenditure caused by metabolic syndrome [42]. Indeed, VC exposure here and in previous work was sufficient to enhance weight gain caused by HFD. It is likely that protecting mitochondria with Aldehyde-1 was the mechanism by which weight gain caused by HFD was attenuated (Fig. 2). The salient findings of the current study support this hypothesis.

We have demonstrated here that activating mitochondrial ALDH2 with Aldehyde-1 was sufficient to confer histological improvement against both HFD and the interaction between HFD and VC (Figs. 3-5). ALDH2 is an important mitochondrial enzyme involved in several cellular processes, notably in the oxidation of aldehydes. ALDH2 dysfunction causes reactive aldehyde accumulation in the mitochondria and damage to this organelle [43]. The active site of ALDH2 contains a thiol group which is sensitive to oxidative modification, making ALDH2 prone to oxidative inactivation by the same species that it metabolizes. For example, endogenous aldehydes, such as MDA and 4-HNE are known inhibitors of ALDH2 [44]. Likewise, we have demonstrated here that CAA, a VC-derived aldehyde, is also a direct inhibitor of ALDH2 (Fig. 7). It is therefore likely that CAA reacts with ALDH2 at its catalytic site, affecting its enzymatic activity. Inhibition of ALDH2 appears to be a shared mechanism between HFD and VC exposure; the combination of HFD and VC further decreased ALDH2 activity in this model (Fig. 1B).

Previous studies have identified that ALDH2 induction/activation is sufficient to protect against oxidative damage to the mitochondria, as well as subsequent cell/organ damage. For example, it was shown that ALDH2 induction was protective in experimental cardiac ischemia/reperfusion in rats by ensuring continuous detoxification of oxidative stress-induced cytotoxic aldehydes [23]. Aldehyde-1 binds to the entrance of

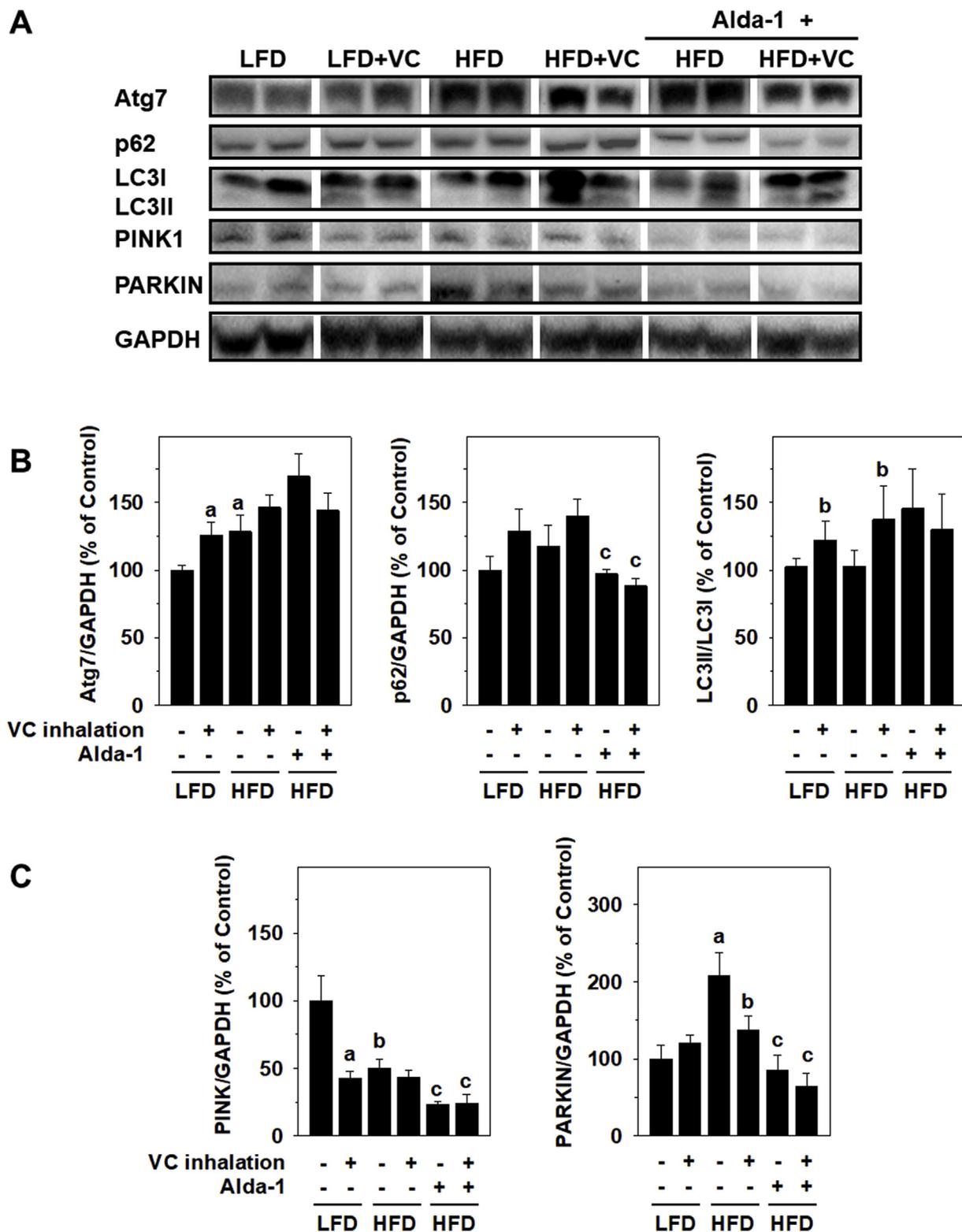


Fig. 6. Dual role of ALDH2 in autophagy regulation in the interaction of HFD and VC. **A:** Representative Western blot analyses for autophagy associated proteins in whole liver extracts: ATG7, p62, LC3I/LC3II, PINK1 and PARKIN. **B:** Densitometric analysis for general autophagy markers ATG7, p62, and LC3II are shown. **C:** Densitometric analysis for mitophagy related markers PINK1 and PARKIN are shown. ^a, $p < 0.05$ compared to LFD control, ^b, $p < 0.05$ compared to absence of VC, ^c, $p < 0.05$ compared to absence of Alda-1. Samples size per group $n = 8-10$.

the active site without interfering with its catalytic residue, thus permitting substrate binding and facilitating the catalytic product release and protecting enzymatic activity [45,46]. Given the impact of VC on mitochondrial oxidative function, we investigated the potential protective effect of Alda-1 against the interaction between VC and HFD in

our model (Fig. 1B). Indeed, Alda-1 significantly blunted liver injury and inflammation caused by VC+HFD (Fig. 3). Moreover, increases in indices of oxidative stress (MDA staining and quantitative TBARS levels) caused by HFD feeding and VC was blocked by ALDH2 induction (Fig. 4).

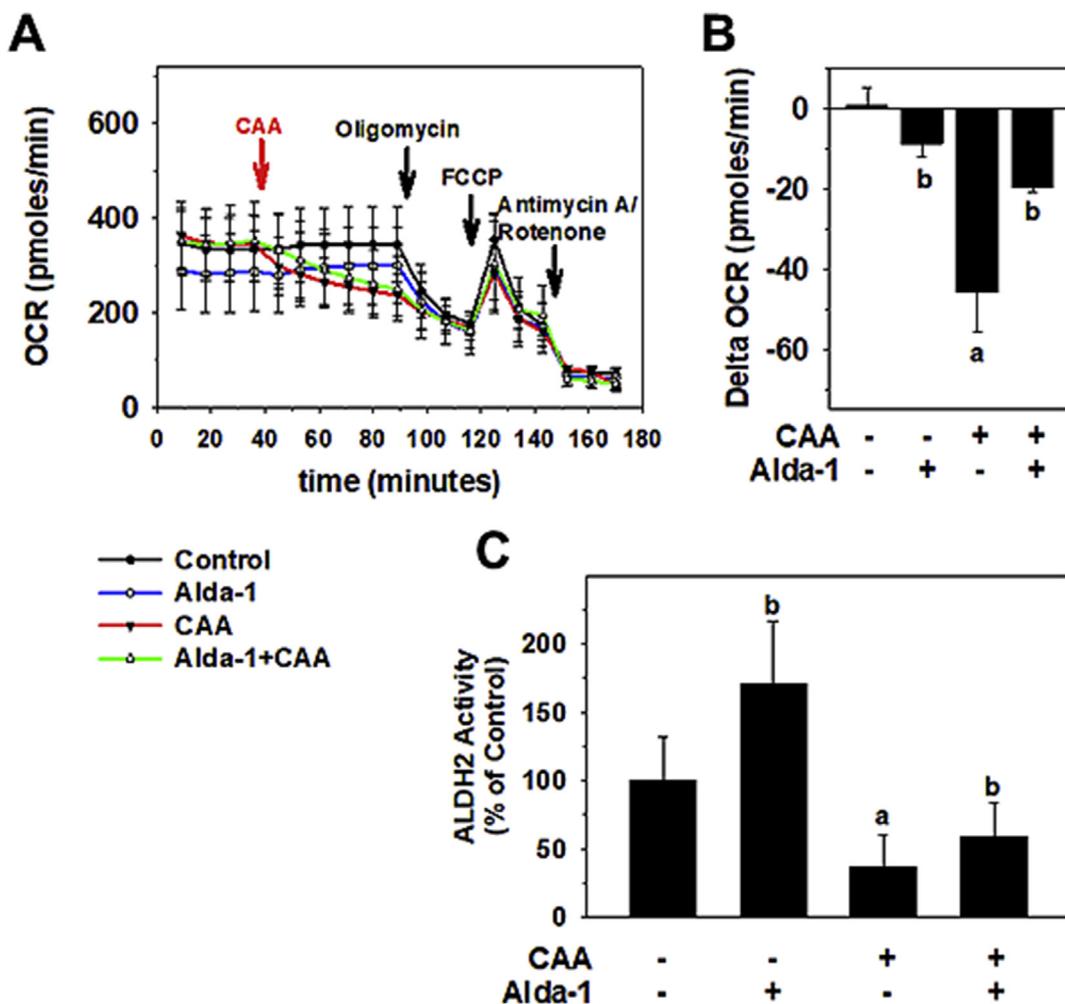


Fig. 7. Alda-1 protects mitochondrial function from dysregulation by VC metabolite chloroacetaldehyde (CAA). A: Primary mouse hepatocytes were isolated and treated \pm Alda-1, \pm CAA as described in Material and Methods. Oxygen consumption rates (OCR) of primary mouse hepatocytes were measured via Seahorse analysis. B: Delta OCR at the first measured time point after CAA addition was calculated and depicted as pmoles/min. C: ALDH2 activity was measured using purified protein \pm Alda-1 \pm CAA. ^a, $p < 0.05$ compared to control, ^b, $p < 0.05$ compared to absence of Alda-1. Samples size per group $n = 8-10$.

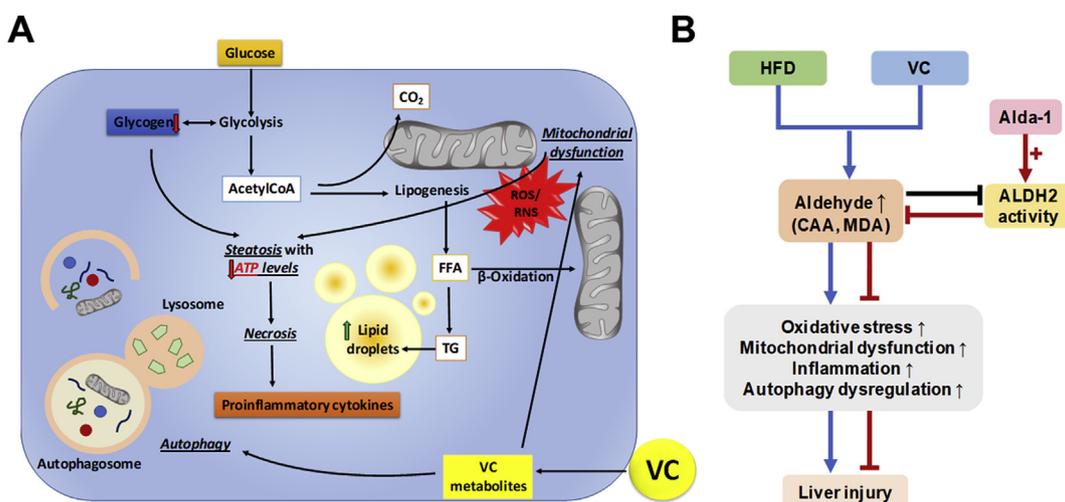


Fig. 8. Working hypothesis. A: Upon exposure to VC, reactive intermediates form through bio activation processes and diet-induced obesity decreases their elimination. Through carbonyl stress and the generation of reactive oxygen and nitrogen species (ROS/RNS), VC metabolites cause mitochondrial damage, which impairs oxidative phosphorylation; the cell increases flux through glycolysis to compensate for this loss of ATP yield. The increased demand for glucose depletes glycogen stores. AcetylCoA is being shunted to lipid synthesis (causing steatosis) rather than β -oxidation, even under conditions of ATP depletion. The combined metabolic stress of VC exposure and HFD creates a 'vicious cycle' that is hypothesized to propagate injury, by enhancing cellular damage and death, thereby increasing an inflammatory response. During the stress response to VC, general autophagy is increased for the clearance of damaged components such as misfolded or adducted proteins, while mitophagy is impaired, likely to conserve remaining energy metabolism.

Mitochondria are crucial for the maintenance of cellular energy homeostasis and possess a 'bioenergetic reserve capacity' that allows this organelle to dynamically respond to changes in metabolic demand. Previous studies have shown that endogenous aldehydes cause a loss of mitochondrial 'bioenergetic reserve capacity'. Impairing this capacity limits the ability of this organelle to serve increased energy demands for maintenance of organ function, cellular repair or detoxification of reactive species [47]. HFD-induced FFA production inhibits glycogen synthesis [48], leading to energy depletion and lipid accumulation (causing steatosis) [16]. In the VC model, this impairment of mitochondrial function further dysregulates carbohydrate and lipid metabolism, causing enhanced glycogen depletion and steatosis (Fig. 5). Alda-1 blocked this effect and resulted not only in improved hepatic levels of glycogen and lipids (Fig. 5) but also in improved glucose tolerance (OGTT, Fig. 2). These results are similar to previous work showing that Alda-1 restores normal hepatic lipid levels in a mouse model of alcohol-induced liver injury [49]. Likewise, Zhang et al. [50] observed that Alda-1 improved glucose and glycogen levels via increased activation of AKT/GSK3 signaling pathway resulting in a cardio-protective effect in an experimental diabetes model.

An indirect mechanism by which Alda-1 may protect in this model is by limiting oxidative stress. Oxidative stress is often part of a 'vicious cycle' that is hypothesized to propagate fatty liver diseases, by enhancing cellular damage and death, thereby increasing an inflammatory response to said damage [51]. As previously demonstrated, VC enhanced the accumulation of oxidatively damaged proteins caused by HFD (Fig. 5) [16]. Mitochondrial damage may therefore be both a cause and effect of oxidative stress in hepatocytes and likely plays a key role in the mechanisms by which VC enhanced HFD-induced liver injury (Fig. 8) [18]. During stress responses, cells initiate rapid adaptive protective responses to limit the impact of that stress. Autophagy is such an adaptive response that has recently received attention in the context of NAFLD. Although the role of autophagy in liver injury is still controversial, it is generally recognized as a protective mechanism. Autophagy can mediate stress-induced adaption and damage control, allowing cells to eliminate damaged components such as organelles, proteins and portions of cytoplasm [52]. For example, several groups have demonstrated a protective role of autophagy during hepatic ischemia/reperfusion injury [53–55]. Moreover, a recent study also demonstrated that Alda-1 enhanced autophagy in a model of hepatic ischemia/reperfusion injury by increasing LC3II and p62 degradation [56]. In line with these results, here we found that Alda-1 significantly decreased p62 protein expression in the HFD + VC group (Fig. 6), suggesting increase autophagy to protect from liver injury caused by HFD and VC.

Although an increase in overall autophagy may be acutely protective to the cell, recent studies have indicated that increased mitophagy may impair energy metabolism of the cell and in the long-term be detrimental. Indeed, here, although general autophagy was increased by Alda-1 administration, indices of mitophagy were actually decreased. Moreover, Alda-1 further decreased PINK1 and PARKIN expression, suggesting an inhibition of mitophagy (Fig. 6). Similar results have also been demonstrated by Ji et al. who have shown that ALDH2 activation had a protective effect on cells via suppressing PINK1/PARKIN-mediated mitophagy after ischemia/reperfusion injury [57]. In that study the authors comment on the "dual effects on autophagy" by ALDH2 in ischemia/reperfusion injury [57], suggesting a differential regulation of mitophagy and autophagy by ALDH2.

5. Conclusion and future prospects

These data support and build on our previous study demonstrating that low-dose exposure to VC will enhance HFD-induced mitochondrial dysfunction. Specifically, here we show that HFD + VC impairs ALDH2 function, resulting in an accumulation of endogenous aldehydes and oxidative stress (Fig. 8). Additionally, in vitro experiments on purified

ALDH2 indicate that decreased VC metabolites indeed decrease enzymatic activity, contributing to mitochondrial dysfunction. Moreover, 3-week Alda-1 treatment in our mouse model of chronic VC inhalation reversed liver injury, oxidative stress and metabolic dysregulation pre-established before the treatment, in part via regulating autophagic pathways (Fig. 8).

Mitochondrial DNA release has recently been demonstrated to serve as a biomarker of liver damage in acetaminophen overdose in humans [58]. Should the mechanisms described herein prove to occur in humans, perhaps individuals exposed to VC occupationally and/or environmentally could be screened with approaches that apply this technique. A limitation of the current work is that it utilizes male mice exclusively. Recent studies also indicated that NASH is a sexually dimorphic disease [59]. It is yet to be determined if sex is a biologic variable that impacts the interaction of VC and NAFLD. Future studies should focus on these aspects to fill the gaps in our knowledge.

Taken together, the data shown here demonstrate for the first time that ALDH2 plays a role in liver injury during the interaction of low-dose VC and HFD, suggesting ALDH2 as a potential therapeutic target during this interaction.

Funding

This study was funded by awards from the National Institutes of Health: K01 DK096042, R03 DK107912 to Juliane Beier.

Research was also supported by an Institutional Development Award (IDEA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM113226 and the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health under Award Number P50AA024337. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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